REVIEW

Newer approaches to the diagnosis of early onset neonatal sepsis

U K Mishra, S E Jacobs, L W Doyle, S M Garland

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Accurate and timely diagnosis of early onset neonatal sepsis remains challenging to the clinician and the laboratory. A test with a rapid turnaround time with 100% sensitivity, rather than high specificity, which allows accurate diagnosis and appropriate antimicrobial treatment or which allows antibiotics to be safely withheld in non-infected infants, is desirable. Many potential markers (acute phase reactants, cell surface markers, cytokines) are not routinely available to the laboratory, and most likely combinations of markers will ensure greater diagnostic accuracy. In the future, molecular biology techniques offer the prospect of rapid identification of both pathogens and antimicrobial resistance markers.

Early diagnosis and treatment of the newborn infant with suspected sepsis are essential to prevent severe and life threatening complications. In this era of multidrug resistance, it is mandatory to avoid unnecessary use of antibiotics to treat non-infected infants. Thus rapid diagnostic test(s) that differentiate infected from non-infected infants, particularly in the early newborn period, have the potential to make a significant impact on neonatal care. Various strategies to reduce morbidity and mortality in newborns with early onset sepsis presenting in the first 72 hours of life involve the use of combinations of clinical signs with haematological and serological markers for identification and intervention in babies at risk. Unfortunately, clinical signs are non-specific and often manifest themselves in the absence of a positive culture. This is particularly common in probable early onset neonatal sepsis with obstetric risk factors where receipt of antenatal maternal antibiotics is common. Among the studies we reviewed, positive cultures ranged from 8% to 73% in the diagnosis of potential neonatal sepsis.[1–3] An additional drawback of culture based diagnosis is the 24–48 hour assay time.

The ideal early diagnostic test for infection would have 100% sensitivity (all patients with the disease are always detected by the test) and 100% specificity (all patients without the disease have a negative test result). However, such an ideal test is unlikely ever to be discovered, as most tests are measured on a continuous scale where there is overlap between infected and non-infected infants. One approach to defining cut-off values for infection in such cases is to consider the point at which most infants are correctly classified—that is, those with infection are identified as infected, and those without infection are identified as non-infected. Such an approach might use a receiver operating characteristic curve to determine the point of maximum accuracy. However, for infection, an infant is more likely to suffer if infection is underdiagnosed and not treated, than if infection is over-diagnosed and the infant is treated unnecessarily. Therefore the more desirable characteristic for a diagnostic test for neonatal infection, including early onset neonatal sepsis, is high sensitivity, rather than high specificity.[4] To be able to reduce antibiotic overuse in non-infected babies, a test with 100% sensitivity will allow safe withholding of antibiotics in babies with suspected sepsis. Even if the test is not 100% specific, as long as the specificity is not too low, it will allow some reduction in antibiotic prescribing depending on how low the specificity is and the prevalence of infection in the group of infants at risk. There remains, however, the problem of determining whether a test is truly 100% sensitive. This requires replication of the sensitivity on large numbers of infected babies, as there remains a small but finite possibility of the test missing a true infection, which reduces with an increasing sample size. For example, with a sample size of 30 and an observed sensitivity of 100%, the upper 95% confidence interval for missing a true infection is 9.5%. It is only with sample sizes in excess of 300 that the upper 95% confidence interval for missing a true infection is <1%. Most studies have fewer than 100 subjects, and even fewer with true infections from which to calculate sensitivity precisely.[4]

Isolation of bacteria from a central body fluid is the standard and most specific method used to diagnose neonatal sepsis.[5] Important procedures to improve the sensitivity and specificity of blood cultures include proper skin disinfection before collection, culturing early in the septic episode, taking an appropriate volume of blood per culture, and, if collecting through an existing intravenous device, ensuring a peripheral culture is also collected and, where practical, more than one bottle per episode. This is not always feasible in a very tiny infant.[3]

ANTENATAL DIAGNOSIS

Intra-amniotic infection is an important and potentially preventable cause of preterm births,

See end of article for authors' affiliations

Correspondence to: Professor Garland, Clinical Microbiology and Infectious Diseases, Royal Women's Hospital, 132 Grattan St, Carlton, Victoria 3053, Australia; suzanne.garland@rch.org.au

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Abbreviations: CRP, C reactive protein; GBS, group B streptococcus; IL, interleukin; IL1ra, IL1 receptor antagonist; PCR, polymerase chain reaction; TNF, tumour necrosis factor

early onset neonatal sepsis, periventricular leucomalacia/cerebral palsy, and maternal febrile morbidity. Overt or subclinical intra-amniotic infection is present in at least 50% of extremely preterm births; an inverse relation has been shown between gestational age at birth and both the frequency of micro-organisms recovered from the chorioamnion and histological chorioamnionitis.[6 7] Potential pathogens largely arise from the ascending route and from the maternal endogenous vaginal flora, causing chorioamnionitis. The release of endotoxins and/or exotoxins from micro-organisms results in stimulation and production of inflammatory cytokines, prostaglandins, metalloproteinases resulting in maternal sepsis (chorioamnionitis, septicaemia, postpartum endometritis), fetal loss (extreme prematurity), and preterm delivery (infant prematurity and its consequences, including increased susceptibility to cerebral palsy), in addition to severe neonatal sepsis.[6]

To determine whether a patient in preterm labour has intra-amniotic inflammation, and thus should be delivered rather than treated with tocolytic agents, is a critical decision with clinical implications for both mother and fetus.[8] Early diagnosis of intra-amniotic infection is problematic, however, because clinical signs and symptoms (including preterm labour) tend to be late manifestations of this condition. Furthermore, the available non-invasive diagnostic tests have limited predictive value, or, as in the case of measurement of interleukin (IL) 6, polymerase chain reaction (PCR) tests, or microbial cultures, the results are often delayed and amniocentesis is required. Therefore improved diagnostic methods are needed to identify women and fetuses who may benefit from specific interventions, such as antibiotics or anti-inflammatory agents.[7]

Amniotic fluid tumour necrosis factor α (TNFα) is a marker for the prediction of early onset neonatal sepsis in patients with preterm labour and intact membranes, and a better independent predictor of early onset neonatal sepsis than placental histology or amniotic fluid Gram stain and/or culture. Amniotic fluid TNFα concentration ⩾41 pg/ml had a sensitivity of 82% and specificity of 79% in the prediction of early onset neonatal sepsis.[9] Although a strong association has been found between maternal serum C reactive protein (CRP) concentrations and cytokine concentrations in the amniotic fluid, in general, maternal measurements of CRP alone do not have a high sensitivity in predicting underlying asymptomatic intra-amniotic sepsis and are not recommended.[10] IL1β was the best predictor of vascular extension of chorioamnionitis, and TNFα was the best predictor of the development of severe early onset neonatal infection.[11] Although TNFα is an important mediator in the pathophysiology of septic shock and systemic inflammatory response syndrome, its utility has not been found to be as good as either IL6 or IL8.[12 13]

Intra-amniotic infection and proteomics

Recent advances in proteomics present a new opportunity to examine the global expression of proteins in tissues and fluids. The proteins or peptides that are preferentially expressed and identified in a disease or pathological state are well suited for the development of convenient, rapid, sensitive, and specific diagnostic assays. Proteomics technology was successfully applied to the analysis of amniotic fluid for the identification by surface enhanced laser desorption ionisation (SELDI) technology of women with intrauterine inflammation and at risk of impending preterm delivery.[8] Diagnosis of intra-amniotic infection by proteomic profiling and identification of a unique peptide profile was possible within 12 hours of intra-amniotic inoculation of a micro-organism and could be reliably present before the onset of labour or other clinical signs or symptoms of infection.[7] Proteomic technology to look for protein markers that could reliably predict sepsis in the neonate is currently under investigation.

POSTNATAL DIAGNOSIS

Multiple studies have examined total leucocyte count, immature to total neutrophil ratio, platelet count, and CRP, and shown that these routine investigations either have low sensitivity and specificity or varying delayed responses early in the course of infection.[14] Leucocyte indices and CRP are considered to be ''late'' markers and are not sensitive enough for early diagnosis of neonatal sepsis.[15] However, abnormalities in these markers soon after a birth complicated by clinical signs and obstetric risk factors of sepsis are highly suggestive of early onset neonatal sepsis. Recent investigations have focused on various groups of chemokines, cytokines, adhesion molecules, and components of the immune pathway that could be used as earlier markers to diagnose infection in neonates.

Acute phase reactants

These groups of endogenous peptides are produced by the liver as part of an immediate response to infection or tissue injury. CRP has been extensively investigated,[14] but there has been more recent interest in procalcitonin. Many other acute phase proteins, including α_1 antitrypsin, fibronectin, haptoglobin, lactoferrin, neopterin, and orosomucoid, have been evaluated in relation to neonatal sepsis. Although most markers show significant increases in infected infants, none have been routinely used clinically, either because of their limited diagnostic accuracy or because they have been superseded by better and more sophisticated tests.

CRP is synthesised within six to eight hours of exposure to an infective process or tissue damage, with a half life of 19 hours, and may increase more than 1000-fold during an acute phase response.[16] The ranges of sensitivity and specificity for diagnosis of early onset sepsis ranges are 43–90% and 70–78% respectively.[14] The specificity and positive predictive value of CRP ranges from 93% to 100% in late onset sepsis. Thus CRP is a ''specific'' but ''late'' marker of neonatal infection.[15] CRP as a diagnostic marker in neonates has higher sensitivity and specificity than total neutrophil count and immature to total neutrophil ratio.[17] We have previously reported that the combination of CRP (>10 mg/l) with full blood examination (abnormal film and/or immature to total neutrophil ratio ⩾0.2) and/or gastric aspirate (⩾5 polymorphs/high power field or potential pathogen on Gram stained smear and/or culture of potential pathogen) has a sensitivity of 97%, specificity of 61%, negative predictive value of 98%, and likelihood ratio of 49 for early onset neonatal sepsis.[18]

Procalcitonin is another important acute phase reactant produced by monocytes and hepatocytes which begins to rise four hours after exposure to bacterial endotoxin, peaking at six to eight hours, and remaining raised for at least 24 hours [19] with a half life of 25–30 hours. Several studies have shown that serum procalcitonin concentrations increase appreciably in systemic bacterial infection, necrotising enterocolitis, and during both early and late onset neonatal sepsis.[20] It may be superior to other acute phase proteins, with sensitivity and specificity ranging from 87% to 100%. It may be useful in assessing the severity of infection, following the progress of treatment, and predicting outcomes.[2 20] However, it is not a readily available diagnostic assay in most institutions.

Cell surface markers

Advances in flow cytometric technology have opened up ways of detecting cell surface antigens on blood cells. This technology appears to be superior to conventional immunological assay methods for localising the activated markers to a

specific cell type. Further, as circulating concentrations of cytokines may not necessarily reflect their biological activities, assessing the cellular response to cytokines may be a better way of identifying early immunological response to bacterial invasion.[21 22]

Neutrophil CD11b and CD64 appear to be promising markers for diagnosis of early and late onset infections.[23 24] CD11b is a subunit of the b2 integrin adhesion molecule, normally expressed at a very low concentration on the surface of non-activated neutrophils. There is a 2–4-fold increase in neutrophil CD11b expression in infants with blood culture positive sepsis,[1 24 25] similar to that seen in adults with blood culture positive sepsis. The sensitivity and specificity of CD11b for diagnosing early onset neonatal sepsis are 86.3–100% and 100% respectively.

A recent study assessing two neutrophil (CD11b, CD64) and two lymphocyte surface markers (CD25, CD45RO) showed that CD64 had the highest sensitivity (97%), specificity (90%), and negative predictive value (99%) as a diagnostic marker for early onset neonatal infection both at the onset of infection and 24 hours later.[26] Combining CD64 with IL6 or CRP further enhances the ability to diagnose localised infections and improves the sensitivity and negative predictive value to 100%.[26] There is an increase in the number of lymphocyte (CD3, CD19, CD25, CD26, CD71 and CD69) and neutrophil (CD11b,CD11c, CD13, CD15, CD33 and CD66b) antigens in preterm newborns in response to infection, with increased expression of CD19, CD33, and CD66b. However, the diagnostic utilities of these markers are yet to be evaluated.[27] Furthermore, none of these white cell surface markers are readily available diagnostically.

Granulocyte colony stimulating factor

Granulocyte colony stimulating factor, a mediator produced by bone marrow, facilitates proliferation and differentiation of neutrophils, and has been proposed to be a reliable infection marker for early diagnosis of neonatal sepsis. A concentration $\geqslant$200 pg/ml has a high sensitivity (95%) and negative predictive value (99%) for predicting early onset neonatal bacterial and fungal infections.[4 28]

Cytokines

Cytokines play an essential role in maturation of progenitors in the bone marrow, in innate immunity, and in the maturation of antigen specific adaptive immunity. As antigen specific immunity develops later—for example, at 2 years of age in the case of encapsulated bacteria—neonates initially depend on natural (innate) immunity. This includes phagocytosis (by monocytes, tissue macrophages, and neutrophils), natural killer cells, and humoral mediators (CRP, complement, and transplacentally acquired maternal antibodies). In response to antigens such as bacterial endotoxins,[29] activated tissue macrophages produce TNF and IL1. These proinflammatory cytokines stimulate endothelial cells to express receptors for intercellular adhesion molecule on white blood cells. This initiates the cytokine cascade towards increased production of IL6, IL8, and chemokines.[30] Some bacteria activate epithelial cells directly to produce inflammatory cytokines.

Newborn infants display a higher percentage of IL6 and IL8 positive cells than do adults.[29] There is sharp rise in IL6 concentration on exposure to bacterial products, which precedes the increase in CRP. Umbilical cord blood IL6 has been consistently shown to be a sensitive marker for diagnosing early onset neonatal sepsis, with sensitivities of 87–100% and negative predictive values of 93–100%.[4 31] IL6 has the highest sensitivity (89%) and negative predictive value (91%) at the onset of infection compared with other biochemical markers, including CRP, IL1β, TNFα, and E-selectin, but sensitivity is reduced at 24 and 48 hours (67% and 58% respectively) because IL6 concentrations fall rapidly and become undetectable after 24 hours.[4 32] The combined measurement of IL6 (early and sensitive) with CRP (late and specific) in the first 48 hours of presumed septic episodes improves the sensitivity compared with either marker alone.[4]

IL8 is a proinflammatory cytokine that is predominantly produced by monocytes, macrophages, and endothelial cells,[33] with similar kinetics to IL6.[34] It is produced in response to various stimuli such as polysaccharide and TNF.[33] IL8 is considered to be a highly accurate marker with sensitivities ranging from 80% to 91% and specificities from 76% to 100%. IL8 and IL8 mRNA concentrations are substantially higher in infected than non-infected newborns.[4] The simultaneous measurement of either CRP[35 36] or neutrophil cell surface marker CD11b with IL8 further enhances the diagnostic value in the diagnosis of neonatal sepsis.[1] A recent multicentred randomised controlled trial of 1291 clinically stable infants with clinical signs or obstetric risk factors suggesting early onset neonatal sepsis reported that the combination of IL8 $>$70 pg/ml and/or CRP $>$10 mg/l significantly reduced antibiotic therapy from 49.6% to 36.1% ($p<0.0001$) without missing infections; sensitivity was 80%, specificity 87%, positive predictive value 68%, and negative predictive value 93%.[37]

Furthermore there was no significant difference in missed infections in the group not treated with antibiotics with the control group.

Another group of proinflammatory cytokines often linked with sepsis is the IL1 family, including IL1α, IL1β, and IL1 receptor antagonist (IL1ra), the last of which exists in substantial excess over IL1β during sepsis. The diagnostic usefulness of IL1β is minimal given conflicting reports of both increasing[30] and decreasing[38] concentrations associated with sepsis. In contrast, concentrations of IL1ra have been shown to be consistently increased in septic patients with concentrations of 6–30 μg/l[39 40] compared with concentrations in uninfected neonates of 2–3 μg/l. Furthermore, one study found that IL1ra had increased by 3 and 15 times the concentration in healthy neonates at 4 and 2 days of age respectively before the clinical diagnosis of neonatal sepsis.[40]

TNFα is a proinflammatory cytokine that stimulates IL6 production and has a broad spectrum of biological actions on several types of target cell, both immune and non-immune. Newborns developing early onset infection are born with higher TNFα concentrations than non-infected infants.[41]

Other markers studied over the last few years include adhesion molecules (intercellular adhesion molecule 1, vascular cell adhesion molecule 1, E-selectin, L-selectin)[4] and complement activation products (C3a-desArg, C3bBbP, sC5b-9),[42] which have been found to be significantly increased during sepsis, but require further evaluation for clinical application in the diagnosis of newborn infection.

Molecular genetics

During the past decade an increasing number of reports on the use of nucleic acid amplification techniques such as PCR in the detection of bacterial genomes in blood cultures have appeared. In particular, broad range PCR analysis, which relies on the fact that the bacteria specific 16S rRNA gene is highly conserved in all bacterial genomes, is a useful method for identification of bacteria in clinical samples.[43] Amplification targeting of this 16S rRNA gene is a potentially valuable clinical tool in samples with low copy numbers of bacterial DNA, as this gene is present at 1 to more than 10 copies in all bacterial genomes. The gene also has a number of divergent regions nested within it, so PCR can be targeted for species specific detection of bacteria in clinical samples. For example, using the adaptation of real time PCR, with the LightCycler system, Golden *et al*[44] detected a group B

Table 1 Accuracy of diagnostic tests or combinations of tests for early onset neonatal sepsis

Diagnostic test	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Antenatal				
Amniotic TNFα ⩾41 pg/ml[9]	82	79	47	95
PCR for genomic DNA in amniotic fluid[45]	100	100	100	100
Postnatal				
CRP[15]	60–82	93–96	95–100	75–87
CRP, FBE, gastric aspirate[18]	97	61	53	98
Procalcitonin[2 20]	82–100	87–100	86–98	93–100
CD11b[24]	96–100	81–100	22–100	100
CD64[23]	64–97	72–96	64–88	84–98
CD64, IL6 or CRP[23]	81–97	71–87	63–74	86–98
GCSF ⩾200 pg/ml[4 28]	95	73	40	99
Umbilical cord IL6[4 31]	87–90	93	93	93–100
IL6[4 32]	67–89	89–96	84–95	77–91
IL6 and/or CRP[4 32]	93	88–96	86–95	95
IL8[4 37]	80–91	76–100	70–74	91–95
IL8 and/or CRP[37]	80	87	68	93
PCR for genomic DNA in blood culture[44]	100	100	100	100

PPV, Positive predictive value; NPV, negative predictive value; TNFα, tumour necrosis factor α; CRP, C reactive protein; FBE, full blood examination; GCSF, granulocyte colony stimulating factor; IL6, interleukin 6; IL8, interleukin 8; PCR, polymerase chain reaction.

streptococcus (GBS) specific *cfb* gene target DNA sequence in blood specimens, reporting a 100% sensitivity and 100% specificity when tested against 26 non-GBS culture detected bacteraemic episodes. The test was capable of detecting 100 copies or 10 pg GBS genomic DNA. This technology has also been reported to be a very sensitive (100%) and rapid method for detecting potential pathogens in amniotic fluid commonly involved in the pathogenesis of preterm labour and adverse neonatal outcome.[45]

However, the performance of broad range PCR analysis at a level of high analytical sensitivity is complex and remains one of the most challenging PCR applications in the diagnostic laboratory. For example, as 16S rRNA gene amplification targets all bacterial species, small amounts of inherent residual DNA present in reagents may be co-amplified, resulting in false positivity. Methods for the removal of potential background contamination include long wave UV light gamma irradiation DNAse, restriction endonuclease digestion, ultrafiltration, and low DNA polymerases. However, many of these methods result in a reduced sensitivity in detecting target DNA, with a detection limit range of 10^3–10^4 copies/ml, which is not ideal for diagnosing sepsis in clinical settings. We have found that a combination of pre-PCR culture with the use of AmpliTaq Low DNA achieves an acceptable level of sensitivity (5–50 copies/ml in a turnaround time of eight hours) for the real time amplification of bacteria in blood samples, without the need to remove any inherent DNA contamination (unpublished work). Consequently it is critical to ensure that high standards and appropriate evaluations of analytical as well as clinical sensitivity are met, if such methods are used in diagnostic laboratories. It is also advisable that all positive broad range PCR products are identified, preferably by a sequenced based method.[43]

Detection by PCR does not result in the antimicrobial sensitivity pattern of the pathogen. However, amplification of known resistance genes allows quick identification of bacteria that are resistant to specific drugs—for example, methicillin resistance for staphylococcal species. With the application of real time PCR, DNA isolation can be accomplished in as little as 20 minutes.[46] Early exclusion of bacterial infection could help to reduce overuse of antibiotics. It is predicted that eventually real time PCR combined with DNA MicroArray technology will allow not only identification of the organism but also the antimicrobial sensitivity pattern, which is so critical to clinical care.

SUMMARY

Table 1 provides a summary of the sensitivities, specificities, and positive and negative predictive values for various markers of early onset sepsis. It was not possible to calculate all values from the data provided in the individual papers.

CONCLUSIONS

The cost associated with preterm birth is substantial in economic, social, and emotional terms. Upper genital tract infection is important in the mechanism of preterm birth, although it is usually asymptomatic. Furthermore, the pathogens associated with intra-amniotic sepsis are those usually involved in early onset neonatal infections. To ensure accurate diagnosis and appropriate antimicrobial management, highly sensitive markers predictive of neonatal sepsis and with a rapid turnaround time are required. Although many putative markers (acute phase reactants, cell surface markers, cytokines) are reported in various clinical research settings, most are not available to the routine diagnostic laboratory. Furthermore, the greatest predictability usually results from a combination of assays. Ultimately, application of the various tools of molecular biology, such as MicroArray chip technology, should allow rapid identification of potential pathogens together with antimicrobial resistance markers.

Authors' affiliations
U K Mishra, S E Jacobs, L W Doyle, Newborn Services, Royal Women's Hospital, Melbourne, Victoria, Australia
L W Doyle, S M Garland, Department of Obstetrics and Gynaecology, and Paediatrics, University of Melbourne, Melbourne
S M Garland, Department of Microbiology and Infectious Diseases, Royal Women's and Royal Children's Hospitals, Melbourne

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